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14. ABSTRACT Fragile X Syndrome (FXS) is a neurodevelopmental disorder characterized by deficits including impaired sensory processing and increased susceptibility to seizure. FXS is caused by loss of function of Fragile X Mental Retardation Protein (FMRP). Because FMRP is highly conserved across animals, we have sought to determine whether decreasing FMRP expression in Xenopus may help understand consequences of loss of FMRP. We established a highly sensitive quantitative in vivo imaging assay to evaluate molecular genetic strategies to decrease FMRP expression in brain neurons and demonstrated the capacity to rescue the decreased FMRP expression by gene delivery. We characterized an innate visually-guided avoidance behavior in tadpoles and showed that the avoidance behavior shows rapid and long-lasting improvement after brief periods of training. Decreasing FMRP expression does not significantly impair visual avoidance behavior. We developed a second behavioral assay to evaluate the loss of FMRP in which animals are exposed to seizure-inducing drugs. Decreased FMRP expression increases seizure latency, which was partially compensated by gene delivery of an FMRP homolog. These studies demonstrate promise in developing Xenopus as a system to study FXS.					
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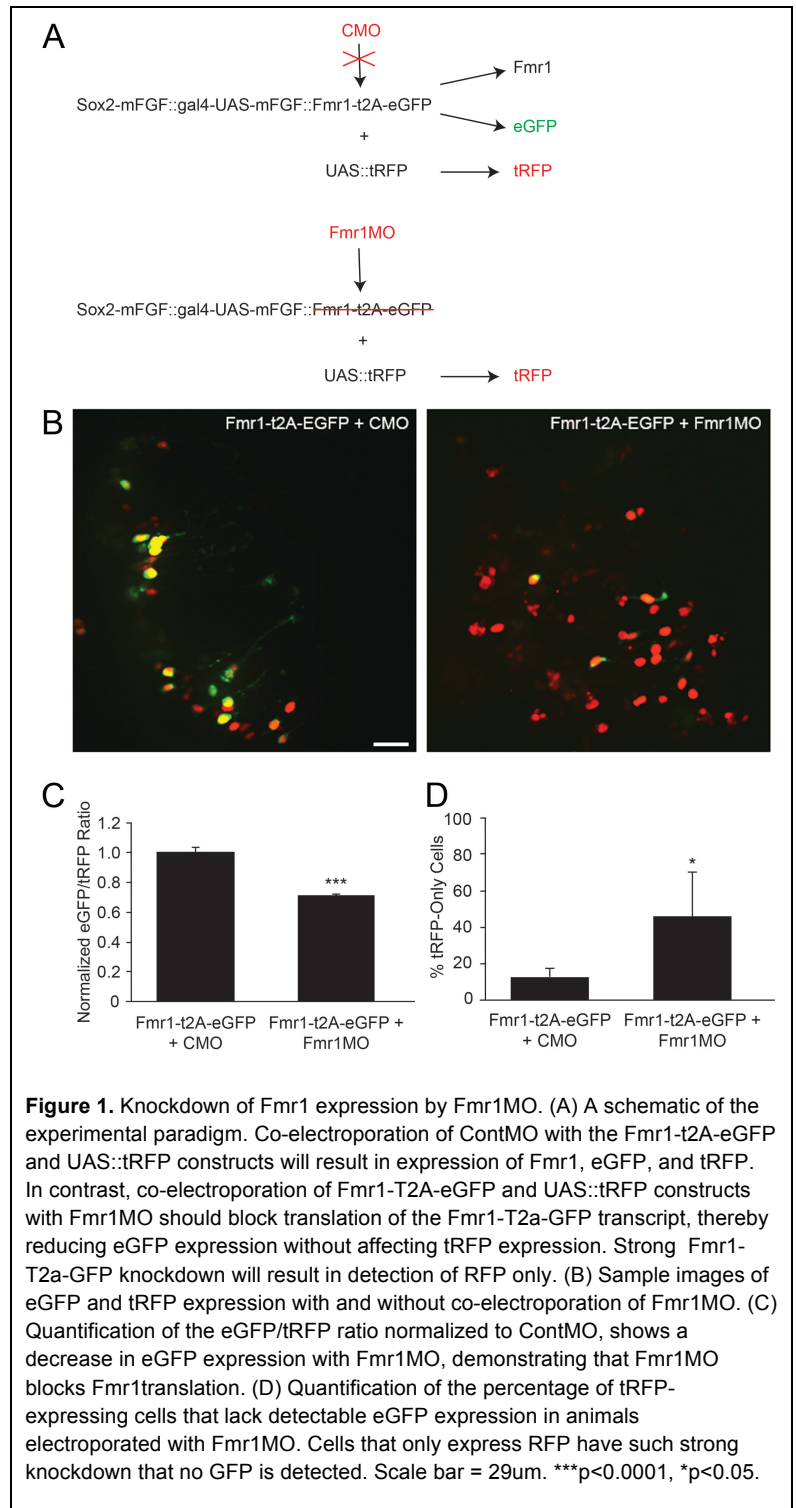
Introduction

Fragile X Syndrome is a neurodevelopmental disorder affecting 1 in 4000 males and 1 in 6000 females worldwide and is the leading cause of inherited intellectual disability. Fragile X Syndrome can also include autistic behavior, heightened sensitivity to sensory stimulation, and seizure. Fragile X Syndrome is caused by mutations in the Fragile X Mental Retardation 1 (FMR1) gene that prevents expression of its protein product, Fragile X Mental Retardation Protein (FMRP). A vertebrate experimental system in which loss of function of FMRP results in behavioral deficits has not been established. Such a system could be valuable to understand mechanisms contributing to deficits in Fragile X Syndrome. We sought to establish *Xenopus laevis* as a model system to study consequences of loss of FMRP on brain function and behavior, and to use this system to identify candidate genes that might rescue behavioral deficits that arise from lack of FMRP. To initiate this project, we established quantitative in vivo imaging methods to knockdown and assay synthesis of FMRP in *Xenopus* tadpole brains. We also established 2 behavioral assays to evaluate the effects of FMRP knockdown. One assay is a visually-guided avoidance behavior, which improves following behavioral training. The other is an assay of response to seizure inducing drug, in which we quantify latency to start of seizure as well as other behavioral parameters. The *Xenopus* tadpole is a unique model system that allows easy access to the nervous system at early stages of development, is amenable to in vivo gene manipulation and gene therapies, and displays behavioral phenotypes that can be altered with genetic manipulations or bath application of drugs that can be absorbed directly through the animal's skin. Progress described below demonstrates that this experimental system may provide insight into Fragile X Syndrome and treatment in people.

Task 1. Test whether knockdown of FMR1 alters tadpole behavior

1a. Validate knockdown of FMR1 by morpholino.

To test whether knockdown of FMRP with the *Fmr1a* morpholino is efficient and specific, we developed a novel assay that provides a sensitive readout of translational knockdown in cells of interest in intact animals. We generated an expression construct that generates a single mRNA of *Xenopus Fmr1b* and eGFP separated by a T2A sequence. The T2A sequence, originally from the insect virus, *Thosea asigna*, induces ribosome skipping and initiation of a second polypeptide with ~100% efficiency when included in the mRNA transcript (Donnelly et al., 2001; Szymczak et al., 2004; Tang et al., 2009). The *fmr1b-t2a-egfp* transcript was expressed under the control of a minimum FGF promoter that includes a Sox2/Oct4 binding domain (bd). Transcription requires binding of endogenous Sox2/Oct4 transcription factors, and therefore results in protein expression in Sox2/Oct4 expressing neural progenitor cells, as described (Bestman et al., 2012). The expression plasmid includes gal4/UAS to amplify *Fmr1-T2A-eGFP* expression, and is designated as pSox2bd::gal4UAS *Fmr1-T2A-eGFP*. The plasmid generates a *gal 4* transcript and a separate transcript *UAS Fmr1-T2A-eGFP*. The gal4 protein binds the UAS sequence and initiates translation. We co-electroporated anesthetized stage 47 tadpoles with 2 expression plasmids, pSox2bd::gal4UAS *Fmr1-T2A-eGFP* and UAS::turboRFP_{nls} (tRFP_{nls}). The tRFP is targeted to the nucleus by the nuclear localization signal (nls) and serves as an internal reference for protein expression driven from the pSox2bd::gal4. We also electroporated an antisense oligonucleotide morpholino against *fmr1a* (Fmr1MO) or a control morpholino (ContMO) at a stock concentration of 0.05mM. The *Fmr1a* morpholino will block translation of the *Fmr1-t2A-eGFP* transcript and will therefore result in decreased expression of both *Fmr1* and eGFP in cells with knockdown without affecting expression of tRFP (Figure 1A). We identified the RFP-expressing cells and determined the proportion of RFP-expressing cells that had no detectable GFP. Animals electroporated with FmrMO had a significant increase in cells expressing RFP_{nls} without detectable eGFP (Figure 1B, D) (RFP+/GFP- cells relative to total RFP+ cells: Fmr1MO 50% ± 8% n=23 tadpoles; ContMO 17% ± 4%, n=20 tadpoles; p<0.05). Since ~50% of RFP+ cells did have



detectable GFP expression we tested whether GFP expression was reduced in animals electroporated with FmrMO, since GFP serves as a proxy for Fmr1 expression because the 2 proteins are synthesized at equimolar quantities. We measured the fluorescence intensities of eGFP and tRFP in each cell, and normalized the ratio of eGFP/tRFP intensities to the average eGFP/tRFP per cell in ContMO animals. In animals electroporated with Fmr1MO, cells had significantly lower eGFP/tRFP ratios than cells from animals electroporated with ContMO (Figure 1B-C) (eGFP/tRFP ratio in ContMO: 1.00 ± 0.03 , $n=391$ cells; Fmr1MO 0.71 ± 0.01 , $n=453$ cells; $p<0.0001$). Together, these results demonstrate that Fmr1MO blocks translation of the Fmr1-t2A-eGFP transcript, resulting in fewer cells with eGFP expression (and by proxy, Fmr1 expression). In cells that express detectable levels of eGFP, those expression levels are decreased relative to RFP expression. We prepared a construct for rescue of Fmr1 expression that contains *Xenopus* Fmr1b with silent mutations rendering it insensitive to the morpholino (Δ xFmr1). When Δ xFmr1-t2A-eGFP was co-electroporated with Fmr1MO, we detected no decrease in the eGFP/tRFP ratio compared to ContMO, confirming that it is insensitive to the morpholino and can be used to rescue knockdown of Fmr1 in our experiments (Δ xFmr1 + CMO 1.00 ± 0.03 , $n=335$ cells; Δ xFmr1 + Fmr1MO 1.08 ± 0.03 , $n=304$ cells). The data are presented as average \pm SEM and a Student's T-test was used to determine significance.

1b. Test visually-guided avoidance behavior.

Tadpoles escape from an approaching object. This innate tectally-mediated visually-guided avoidance behavior is assessed as a change in swim trajectory when a moving spot enters the animal's visual field at approximately right angles to the eye (Figure 2A). We used the avoidance index (% of avoidance responses per 10 trials) to quantify the avoidance success rate when tadpoles encounter moving spots (Dong et al., 2009; McKeown et al., 2013; Shen et al., 2011). We assessed visual avoidance over 4-24 hours by measuring avoidance during 1-minute test periods with half an hour intervals between tests. We did not observe any habituation of the avoidance index when *Xenopus* was tested for avoidance over 7 hours (Figure 2B), indicating our assay is suitable for studies of behavioral plasticity over this time frame.

To test whether Fmr knockdown can affect an innate behavior, we tested visually-guided avoidance behavior in stage 47 *Xenopus* tadpoles after they were co-electroporated with morpholinos against Fmr1a and Fxr1. Fxr1 is an autosomal paralog of Fmr1a that is highly similar and might be functionally redundant with Fmr1a. To eliminate this possible redundancy, we knocked down both Fmr1a and Fxr1. We

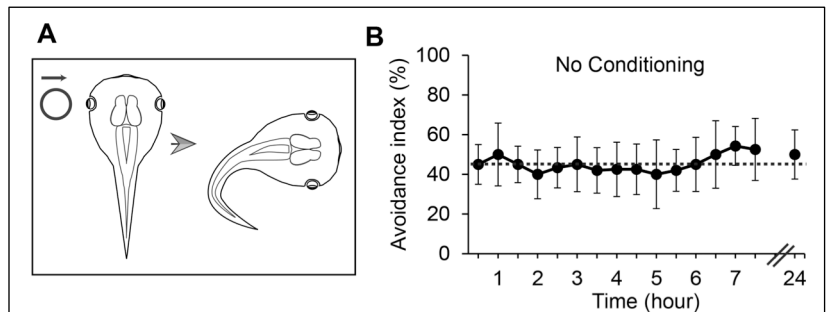


Figure 2. Visual avoidance behavior (A) An illustration of the visual avoidance behavior in response to a moving spot approaching the eye. The tadpole is swimming forward when it encounters a spot moving toward it from left to right (small arrow represents the direction of movement of the spot). In response to the stimulus, the animal changes its swim trajectory by turning sharply to the left or right, called avoidance behavior. (B) The avoidance index in response to 0.4 cm moving spots remains constant over 7 hours when animals are tested for 1 minute every 0.5 hour. The avoidance index remains unchanged after 24 hours. Dotted line is the average of the avoidance index of first three tests. $N=8$ animals.

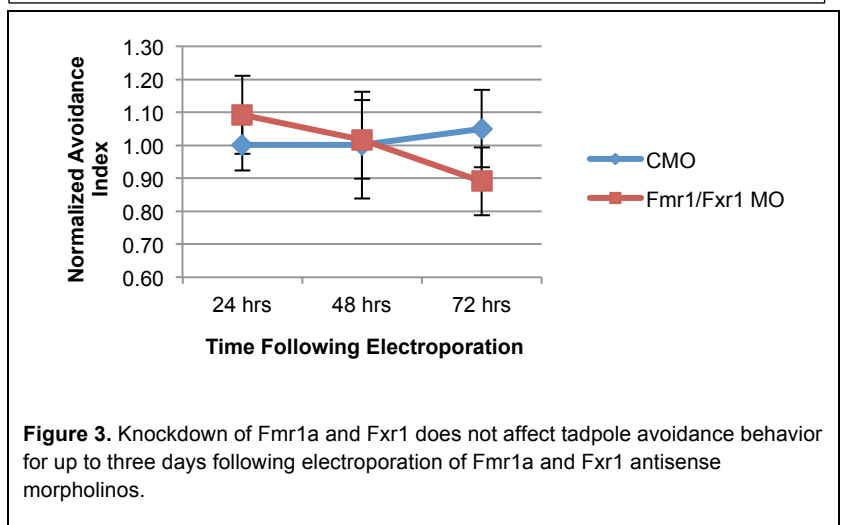
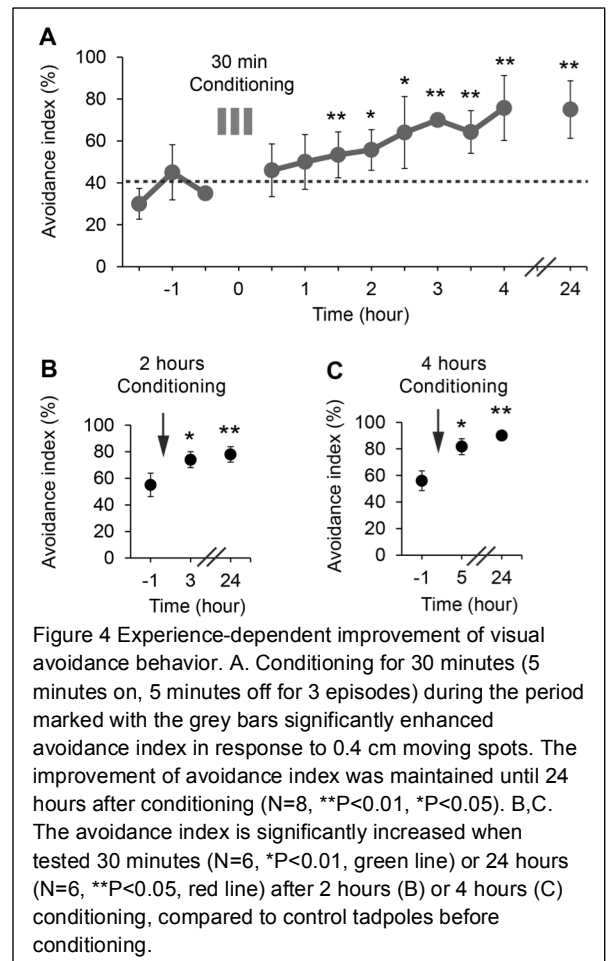


Figure 3. Knockdown of Fmr1a and Fxr1 does not affect tadpole avoidance behavior for up to three days following electroporation of Fmr1a and Fxr1 antisense morpholinos.

screened stage 47 animals for the optomotor response to assess normal swimming behavior. Animals passing the optomotor screen were electroporated with antisense morpholinos against *Fmr1a* and *Fxr1* (Fmr1/Fxr1MO), or control morpholino (ContMO), at a stock concentration of 0.05mM. One, two and three days later, tadpoles were placed in a clear tank and randomly moving dots were presented for 90 sec using a microprojector positioned below the tank, as described (Shen et al., 2011). Videos of tadpole movements were recorded and analyzed for encounters with dots approaching the eye perpendicularly. Only animals having at least 5 encounters during the 90 sec exposure period were included in the analysis. The percent of encounters that gave a turning response within 500ms of the encounter was called the Avoidance Index. To control for clutch to clutch variation in animal behavior, Avoidance Indices for each group and time point were normalized to the average Avoidance Index of the matched control group taken one day after electroporation for each experiment. We found no significant effect of *Fmr1a* and *Fxr1* knockdown on the Avoidance Index at the time points tested (Figure 3) (24 hrs: ContMO 1.00 ± 0.08 vs Fmr1/Fxr1MO 1.09 ± 0.12; 48 hrs: ContMO 1.00 ± 0.16 vs Fmr1/Fxr1MO 1.02 ± 0.12; 72 hrs: ContMO 1.05 ± 0.12 vs Fmr1/Fxr1MO 0.89 ± 0.10). The data are presented as average ± SEM and a Student's T-test was used to determine significance.

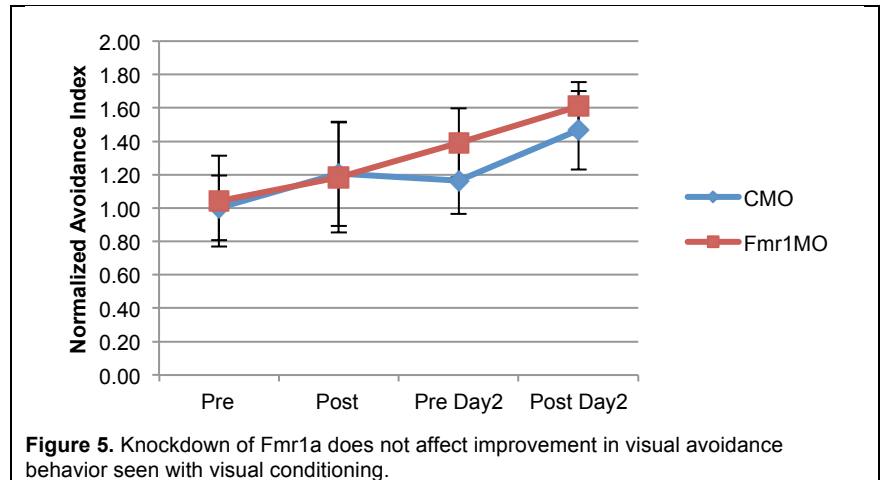
1c. Test improvement of visually-guided avoidance behavior with training.

We tested the effects of several different protocols for visual conditioning on the innate tectally-mediated visual avoidance behavior (Figure 4). We exposed freely swimming animals to a stimulus composed of bars moving at 0.3 Hz in 4 directions in pseudorandom order and tested the visual avoidance index in response to moving spots of 0.6, 0.4, 0.2, and 0.1 cm in diameter. Previous experiments showed that the visual avoidance response is maximal for 0.4 cm moving spots (Dong et al., 2009; Shen et al., 2011). Exposure to 30 minutes of conditioning consisting of 3 five-minute episodes of moving bars with 5-minute intervals without stimulus between episodes resulted in long-lasting enhancement of the behavioral response (Figure 1C). The avoidance index was measured three times at 30-minute intervals to establish a baseline avoidance index before tadpoles were exposed to the visual conditioning. The avoidance index was determined every 30 minutes over the next 4 hours to evaluate the effect of conditioning. A significant increase in the avoidance index was detected 1.5 hour after 30 minute of visual conditioning. The improvement of the avoidance index was maintained for 24 hours (Figure 1C). Exposure to for 2 or 4 hours of continuous visual conditioning (VC) significantly improved the avoidance response when tested 30 minutes or 1 day after the end of the conditioning period (Figure 1D, E). Visual conditioning did not significantly affect responses to other spot sizes (Supplementary Figure 1A, 1B). These results indicate that the visual avoidance response is plastic in response to brief exposure to visual conditioning, that the plasticity can be detected shortly after conditioning and is maintained for at least one day. We used this conditioning protocol in the following experiments investigating the mechanisms underlying visual avoidance plasticity.



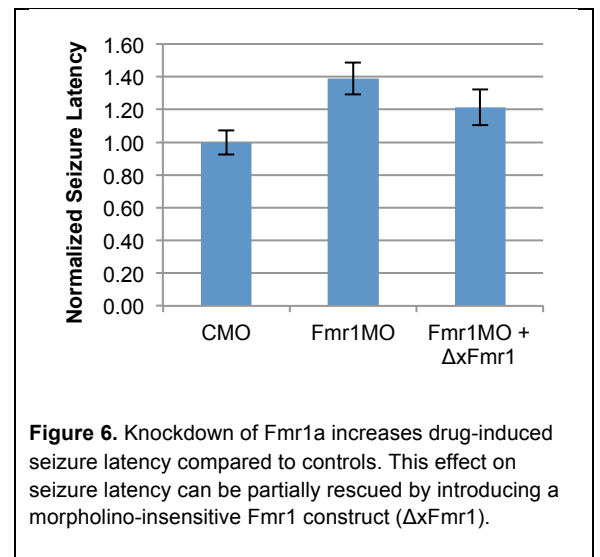
We tested whether knockdown of Fmr1a will affect the ability of animals to improve performance in the avoidance assay following short term visual enhancement (STVE) provided by exposure to a simulated motion stimulus comprised of rows of LEDs that are sequentially turned on and off. Stage 47 animals were screened for the optomotor response. Animals passing the optomotor screen were electroporated with an antisense morpholino against Fmr1a (Fmr1MO), or control morpholino (CMO), at a concentration of 0.05mM. 3 days following electroporation, animals were

presented with moving dot stimuli for 90s as described above. Then, animals were presented with STVE for 4 hours. Following STVE, animals were presented once again with the moving dot stimuli for 90s. This moving dot-STVE-moving dot paradigm was repeated again 24 hours later. Videos of tadpole movements were recorded and the Avoidance Index was quantified as described above. To control for clutch to clutch variation, Avoidance Indices were normalized to the average Avoidance Index of the ContMO group before STVE for each experiment. We found no significant effect of Fmr1 knockdown on STVE-induced improvement in the Avoidance Index (Figure 5) (Pre STVE Day 1: CMO 1.00 ± 0.19 n=12, Fmr1MO 1.04 ± 0.27 n=12; Post STVE Day 1: CMO 1.21 ± 0.31 n=12, Fmr1MO 1.18 ± 0.33 n=6; Pre STVE Day 2: CMO 1.16 ± 0.2 n=11, Fmr1MO 1.39 ± 0.21 n=10; Post STVE Day 2: CMO 1.47 ± 0.23 n=11, Fmr1MO 1.61 ± 0.15 n=6). The data are presented as average \pm SEM and a Student's T-test was used to determine significance.



1d. Test susceptibility to drug-induced seizure.

We tested whether knockdown of Fmr1a alters susceptibility to PTZ-induced seizure. Stage 41-43 animals were electroporated with antisense morpholino against Fmr1a (Fmr1MO) or control morpholino (CMO). Three days later, animals were exposed to 15mM PTZ in rearing solution for 20 min. Videos of tadpole movements were recorded every 2 min for 30 sec and analyzed post hoc. The latency between drug exposure and onset of seizure, defined as a C-shaped body contraction, was quantified. To control for clutch to clutch variation, seizure latencies were normalized to the average control seizure latency for each experiment. We found that Fmr1 knockdown significantly increased seizure latency compared to CMO (Figure 6) (CMO 1.00 ± 1.07 n=57, Fmr1MO 1.39 ± 0.10 n=56, $p=.01$). The data are presented as average \pm SEM and statistical significance was determined by ANOVA followed by a Tukey-Kramer test.



1e. Test rescue of FMR1A knockdown with FMRP.

We found that co-electroporation of Fmr1MO with a construct containing *Xenopus* Fmr1b with a silent mutation rendering it insensitive to morpholino (Δ xFmr1), could partially rescue the seizure latency defect caused by Fmr1a morpholino (Figure 6) (Fmr1MO + Δ xFmr1 1.21 ± 0.11 n=45). The data are presented as average \pm SEM and statistical significance was determined by ANOVA followed by a Tukey-Kramer test.

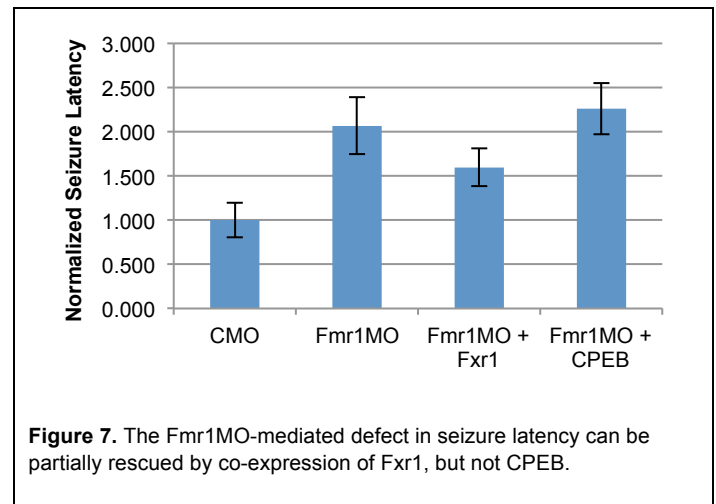
Task 2. Test rescue of FMR1A knockdown with 4 candidate genes: CPEB, pumilio, staufen, Fxr1.

2a. Test visually-guided avoidance behavior and improvement with training.

We found no defects in visual avoidance behavior or training-induced improvement in visual avoidance with knockdown of Fmr1a.

2b. Test susceptibility to drug-induced seizure.

To test the ability of other RNA binding proteins to rescue the Fmr1a knockdown-mediated defect in seizure latency, we co-electroporated Fmr1MO with expression constructs for either *Xenopus* Fxr1 or *Xenopus* CPEB. Stage 42-43 animals were electroporated with antisense morpholino against Fmr1a (Fmr1MO) or control morpholino (CMO) at a concentration of 0.05mM. A subset of animals electroporated with Fmr1MO were also electroporated with expression constructs for Fxr1 or CPEB. Three days later, animals were exposed to 15mM PTZ in rearing solution for 20 min. Videos of tadpole movements were recorded and analyzed for seizure latency as described above. As shown above, we found that Fmr1 knockdown significantly increased seizure latency compared to CMO (CMO 1.00 ± 0.2 n=7, Fmr1MO 2.07 ± 0.32 n=8, $p < 0.05$). Furthermore, we found that co-electroporation of Fmr1MO with Fxr1 partially rescued Fmr1 knockdown (Fmr1MO + Fxr1 1.60 ± 0.22 n=6, $p = 0.437$ compared to CMO), while co-electroporation of Fmr1MO with CPEB had no effect on seizure latency (Fmr1MO + CPEB 2.26 ± 0.29 n=6, $p < 0.05$ compared to CMO) (Figure 7). The data are presented as average \pm SEM and statistical significance was determined by ANOVA followed by a Tukey-Kramer test.



Key Research Accomplishments

- Establish a quantitative in vivo imaging assay to evaluate protein knockdown in neurons
- Develop and test reagents to manipulate FMRP protein expression in vivo
- Document decreased FMRP synthesis and rescue of loss of protein by gene therapy
- Establish quantitative behavioral assays to evaluate consequences of decreased FMRP expression or rescue by gene delivery methods
- Demonstrate improvement in visual avoidance behavior in response to training
- Demonstrate changes in behaviors in response to decreased FMRP synthesis and rescue by gene delivery

Reportable Outcomes

- A manuscript is in preparation which includes some of the work accomplished with this DoD support.

Conclusion

This study has resulted in significant progress toward our goal of establishing *Xenopus* as a model system to study Fragile X Syndrome and to identify candidate genes, as future drug targets that may be effective therapies for treatment. Over the past few years, several candidate treatments for Fragile X Syndrome have gone to clinical trials. Though promising, no treatment has yet been approved. This sad news indicates that dramatically different approaches to identifying candidate therapeutics are worth investigating.

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Appendix

None